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Original Article

Pharmaceutical Development and Microbiological Quality Control of Cosmetic Formulations Developed at a Laboratory School in the City of Sobral, Ceará, Brazil.

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Abstract: Emulsions are used for skin care applications. That's why it's important to evaluate the stability of these products in order to ensure their physicochemical and microbiological chracteristics. The stability study aims to provide an indication of the product's behavior in a given time interval, compared to the temperature and humidity conditions to which it may be subjected. This study aims to evaluate the microbiological quality of anionic and non-ionic emulsions, produced in the Pharmacy School of INTA College. Two kilograms of each emulsion were produced, which were divided into two storage containers. One of them was placed at room temperature and the other in an oven at 40 °C ± 2 °C for 60 days. Samples were analyzed for their microbiological characteristics (counting of mesophilic aerobic microorganisms as well as fungi and yeast counting).- Analysis were performed after its manufacturing (T0), 30 days after manufacturing the emulsion (T1) and 60 days after (T2). The microbiological analysis found out that only one sample was not according to the Brazilian law RDC 481 of September 23, 1999, with an average of Colony Forming Units (CFU) above the established limit. The high growth of micro-organisms in this sample was attributed to inadequate conditions during test procedure, which were accomplished without the use of a laminar flow.
Keywords: emulsions, quality control, microbiological testing, microbial colony counting.

Introduction

Emulsions are thermodynamically unstable systems consisting of two immiscible phases, i.e., a dispersed phase inside another one in the form of finely divided small droplets, in which the diameter ranges from 0.1 and 10 micrometers. The dispersed phase (or internal) is the phase that is presented in the form of drops whereas the dispersing phase forms the matrix in which these droplets are dispersed. Emulsifiers agents, like octyl stearate, cocamide propylbetaine and benzalkonium chloride, are used to stabilize these two phases [1]. The emulsifiers are added to the emulsions in order to increase the stability of the emulsions. They act by reducing the interfacial tension, facilitating the formation of small drops and even preventing preexisting droplets from getting together into larger drops by the coalescence phenomena [2].

The development of new products for the pharmaceutical and cosmetic markets has increased significantly in the recent years; there are many dermatological bases that, when incorporated into pharmaceutical preparations, may give rise to innovative medicines or cosmetics. The emulsions have been used both for the incorporation of drugs, as well as cosmetic actives [3,4].

The most common emulsion preparation is the oil-in-water type (O/W). This type of formulation penetrates quickly into the skin, and is also easily removed from the it. For this reason, these emulsions are more indicated for topical use since it doesn't present a greasy sensation [5]. Whatever the purpose of the emulsion is, it should remain pharmacotechnically stable within a fixed period, in relation to pH, viscosity, spreadability and organoleptic characteristics. However, despite the technical precautions taken during the production of emulsions, alterations may occur within a certain period of time after its production [6].

The barrier membrane in human skin has an important function that is crucial to limit the body's water evaporation. It is also useful to prevent the entry of exogenous chemicals. However, there is a transepidermal water loss of about 100-150 mL per day through healthy skin. The skin barrier function is assured by the outer layer of the epidermis, the stratum corneum. The hydration of the *stratum corneum* is crucial in regulating the skin barrier properties [7,8,9].

Under normal conditions, the water supply for skin hydration and transepidermal water loss is regulated within the body. At steady state, the *stratum corneum* is hydrated to a level that is determined by the water gradient across the skin, and its hydration determines the permeability of the stratum corneum [8,9]. The *stratum corneum* is considered the major barrier to the penetration of drugs through the skin. It is essential that the formulation guarantees an efficient penetration through the stratum corneum barrier. [10,11]. Moisturizers are an important part of the dermatologist's armamentarium to treat dry skin conditions and maintain healthy skin. Among the different cosmetics, the emulsions are widely used for hydration of dry skin [12].

The purpose of stability testing is to provide information on the changes of physicochemical and microbiological characteristics, of the product as time goes by, under the influence of some environmental factors such as temperature, humidity and light. These tests provide information that helps to define the shelf life of pharmaceuticals and cosmetics [13]. The stability of pharmaceutical products depends on environmental factors such as light, humidity, temperature, and others related to the product itself, such as physicochemical properties of active ingredients and pharmaceutical excipients, pharmaceutical dosage form and composition, production methods, and properties of packaging materials [14].

Regarding the chemical stability of each active ingredient, these should maintain its integrity and chemical potency established in the package. Among the physical characteristics, phase separation is critical, because all other properties of the emulsion might be affected, such as viscosity, spreadability and organoleptic characteristics, because of loss of homogeneity of the product. Regarding the microbiological stability, non-sterile products must have components that do not allow microbial growth. The main stability studies performed to emulsions are the physical and microbiological stability [15].

To decrease the time which is required by stability testing, the samples are subjected to extreme conditions that stress the stability of the product. For example, you can determine the physicochemical stability of cosmetic formulations submitting them to stress conditions such as high temperature and humidity conditions [16]. The ANVISA (National Health Surveillance Agency) created in 2004 the Cosmetic Products Stability Guide, which establishes the temperature and humidity conditions to perform accelerated stability studies [17].

The purpose of microbiological evaluation of cosmetics is to count the number of viable microorganisms and ensure the absence of pathogenic microorganisms. Microbial growth in emulsions may affect

some physicochemical characteristics of the product, may cause changes in color and odor, changes in pH, emulsion breaking and hydrolysis of oils [5, 18].

The extent of microbial contamination depends largely on contaminated components during the manufacture of the product as well as the place it is manufactured. The microbiological quality control is important to evaluate contamination points and establish norms of control, in order to obtain excellent quality and stability products. Therefore, a microbiological monitoring during manufacturing, packaging, storage of cosmetics is a method to ensure the safety of the consumer's health [17,19,20].

Thus, this study aimed to accomplish the counting of mesophilic aerobic bacteria, fungi and yeasts in one lot of anionic emulsion and one lot of nonionic emulsion, each lot containing two kilograms. Each lot of emulsion was divided in two: half was stored in room temperature and the other half was stored in an oven (40 ºC ± 2 ºC). Microbiological quality of each lot were evaluated by counting of mesophilic aerobic microorganisms, fungi and yeasts. The manufacturing and stability tests were performed in the Pharmacy School and Núcleo de Bioprospecção e Biologia Molecular Aplicada (NUBEM) of INTA College.

Materials and Methods

The emulsions of this study were standardized by the Pharmacy School of INTA College, based on Brazilian Pharmacopoeia and were produced by the conventional method, from the National Form of Brazilian Pharmacopoeia 5th Edition, 2010. The aqueous phase was heated to 80 °C and the oil phase heated to 75 °C. After that, the aqueous phase was slowly poured into the oil under constant stirring and continued until the emulsion reached room temperature (25 °C ± 2 ºC). A total of 2.000 g of each emulsion were prepared, divided into two containers, where one was stored at room temperature and the other stored in an oven (40 ºC ± 2 ºC). The components and their quantities (in percentage) are detailed in Tables 1 and 2.

Table 1: Composition of the nonionic emulsion produced in the Pharmacy School of the INTA College

Components	Amount (%)	Components	Amount (%)	Components	Amount (%)
Aqueous Phase		**Oil Phase**		**Additional Phase**	
Sodium EDTA	0.1	Butylhydroxytoluene	0.1	Imidazolidinylurea solution 50%	0.6
Methylparaben	0.2	Propylparaben	0.1		
Purified water	75.9	Nonionic self-emulsifying wax	16.0		
Glycerin	3.0	Octyl stearate	2.0		
		Cyclomethicone	2.0		

Table 2: Formulation of the anionic emulsion produced in the Pharmacy School of the INTA College.

Components	Amount (%)	Components	Amount (%)	Components	Amount (%)
Aqueous Phase		**Oil Phase**		**Additional Phase**	
Sodium EDTA	0.1	Butylhydroxytoluene	0.1	Imidazolidinylurea solution 50%	0.6
Methylparaben	0.2	Propylparaben	0.1		
Purified Water	69.9	Anionic Self-Emulsifying Wax	16.0		
Glycerin	3.0	Octyl Stearate	8.0		
		Cyclomethicone	2.0		

A batch of each emulsion was produced containing 2.000 grams. Each lot was divided into two parts. Half of each lot was used for the accelerated stability tests and packaged in a sealed container and stored in an oven at 40 ± 2 °C. The other half was stored in a sealed container and stored at room temperature at 25 ± 2 °C.

The formulations were submitted to the conditions cited for a period of 60 days and the analysis were performed on Day 1 (T0), at the 30th day (T1) and the 60th day (T2). The first day of the test corresponds to the day of production of the samples. The conditions of temperatures follow the guidelines of the Cosmetic Products Stability Guide [17].

Neutralization of the inhibitory activity of conservants

Before performing microbiological analysis, tests were done to prove the absence of the inhibitory effect on the growth of microorganisms due to the inhibitory activity of conservants. The evaluation of the inhibitory capacity of the conservants present in the sample is critical to an effective microbiological evaluation. The inhibition of growth of microorganisms due to the bacteriostatic or fungistatic effect of the formulation may cause a false-negative result in microbiological analysis. Inhibition of the conservants was achieved by using two methods: by the addition of 0.4 % polysorbate 80 and decimal serial dilutions. The sample was prepared by using 10 g of each sample in 90 mL of 0.9 % saline, resulting in a 1:10 dilution. Also, 0.4 % polysorbate 80 was added in saline solution to neutralize the parabens and imidazolidinylurea in the formulations[21,22].

The neutralization of the inhibitory effect was performed by using 0.4 % polysorbate 80 and decimal serial dilutions. The growth of the microorganisms tested was observed: *Staphylococcus aureus*, *Salmonella typhimurium*, and *Pseudomonas aeruginosa* in all samples and in the positive control, meaning the inactivation of the inhibitory effect of conservants[21,22].

After the neutralization of conservants, it's possible to perform other microbiological assays with greater security, since it was proven that the conservants of the samples are neutralized and will not result in a false-negative result [21,22].

Microbiological assays

Cosmetic products for topical application admit the presence of microbial load, provided they do not exceed the limits specified by RDC No. 481 of September 23, 1999. Cosmetic products are divided into two types and in each type there is a microbial load limit as described in Table 3.

Table 3: Limits of acceptability of microbial growth in cosmetic products (According to [12])

<table>
<tr><td rowspan="5">TYPE I</td><td rowspan="2">Products for children use</td><td>a) Count of total mesophilic aerobic microorganisms, no more than 10^2 CFU/g or mL</td><td rowspan="5">TYPE II</td><td rowspan="5">Other products susceptible to microbiological contamination</td><td>a) Count of mesophilic aerobic microorganisms, no more than 10^3 UFC/g or mL</td></tr>
<tr><td>Maximum:$5x10^2$ CFU/g or mL</td><td>Maximum:$5x10^3$ CFU/g or mL</td></tr>
<tr><td rowspan="2">Products for eye area</td><td>b) Pseudomonas aeruginosa absence in 1 g or mL</td><td>b) Pseudomonas aeruginosa absence in 1 g or mL</td></tr>
<tr><td>c) Staphylococcus aureus absent in 1 g or ml</td><td>c) Staphylococcus aureus absent in 1 g or ml</td></tr>
<tr><td rowspan="2">Products entering in contact with mucous tissues</td><td>d) Absence of total and fecal coliforms in 1g or ml</td><td>d) Absence of total and fecal coliforms in 1g or ml</td></tr>
<tr><td>e) Absence of Clostridium sulfite reducers in 1g (talcs only)</td><td>e) Absence of Clostridium sulfite reducers in 1g (talcs only)</td></tr>
</table>

The cosmetic formulation developed in this study has the following limits: the counting of aerobic mesophilic microorganisms, fungi and yeasts should not exceed 10^3 CFU/g or mL. The sample must show no *Pseudomonas aeruginosa*, *Staphylococcus aureus* absence and absence of total and fecal coliforms [18]. The tests should employ aseptic techniques during sampling and carrying out of the tests. If the sample has some antimicrobial activity, it must first be removed or neutralized. In the present study, it was verified by testing the inhibitory capacity [21].

Preparation of samples and dilutions

It was used 10 g of the formulation for each sample were used, 90 mL of 0.9 % saline with added 0.4 % Polysorbate 80 to inactivate conservants, forming decimal serial dilutions. The tests were performed on each batch of the formulations [21].

Total mesophilic aerobic microorganisms counting

For the test it was added 15 to 20 mL of soybean-casein agar in Petri plate. After dry, it was sown on the surface of each means 0.2 mL of the sample prepared previously by the spread-plate method [13]. As described in Figure 01, the agar plates containing TSA (tryptone soy agar BD®) were incubated at 37 °C for 24 hours to determine the total number of aerobic mesophilic microorganisms. It was taken the arithmetic mean of the plates, by multiplying

the result by the corresponding dilution and calculated the number of Colonies Forming Units (CFU) per mL of the sample. For the calculation of Colony Forming Units, we selected plates with growth between 25-250 colonies. The plates which grew below 25 colonies were considered for the calculation, however, the result was expressed in estimated CFU [23].

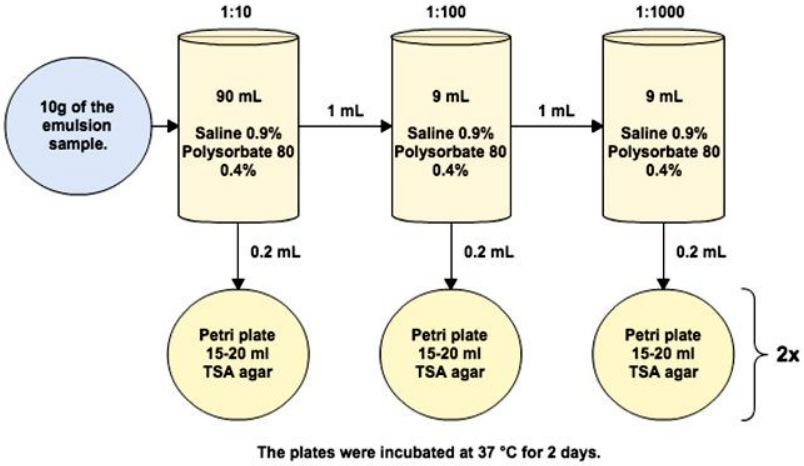

Figure 1: Flowchart of the preparation procedure of dilutions and counting of the number of aerobic mesophilic microorganisms.

Fungi and yeasts counting

To perform the fungi and yeast counts 15 and 20 mL of Potato dextrose agar (Difco®) in Petri plate. After dry, it was sown on the surface of each medium 0.2 mL of the sample prepared previously by the spread-plate method [21]. As shown in Figure 02, the agar plates were incubated at 22.5 °C ± 2.5 °C for 7 days, and after, fungi and yeasts were counted. The arithmetic mean of the counting was taken of the plates and the number of CFU/mL was estimated for each sample.

For the calculation of Colony Forming Units, we selected plates with growth between 25-250 colonies. The plates in which we counted less than 25 colonies were considered for calculation; however, the result was expressed in estimated CFU [23].

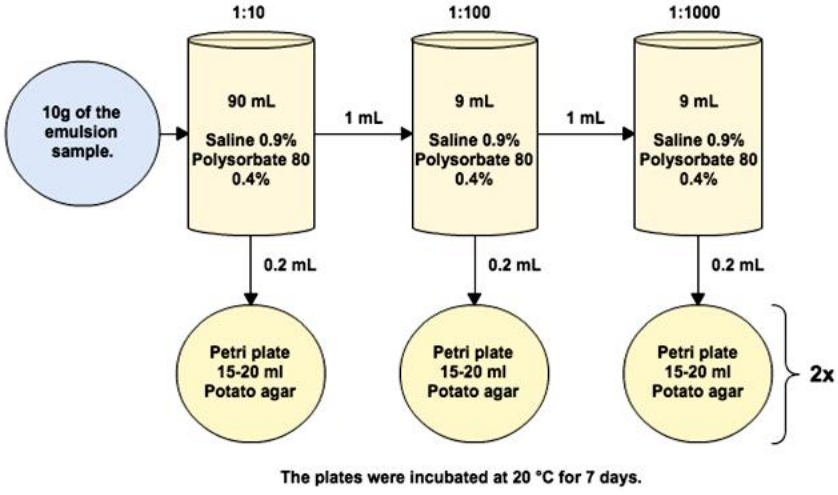

Figure 2: Flowchart of the preparation procedure of dilutions and count of the number of fungi and yeasts.

Results and Discussion

Microbial contamination can compromise product performance due to breach of the stability of emulsions, changes in physicochemical characteristics, as well as lead to inactivation of the active ingredients and excipients present in the emulsion [24]. To avoid microbiological contamination problems, it is necessary to provide proper information on the use and conservation of the product. During preparation, the use of conservants is essential, as well as aseptic methods and disinfection of premises and equipment [17].

Total mesophilic aerobic counting (MAM)

The results of mesophilic microorganisms count are presented in Table 4. The RDC No. 481 of September 23, 1999 states that the average recommended for products for topical application is 1000 CFU/g or mL and the maximum allowed is 5000 CFU/g or mL. In this paper, it was considered within the limits emulsions that presented a quantity to 1000 CFU/g or mL [18].

Table 4: Count of mesophilic aerobic microorganisms in both emulsions after production, 30 days and 60 days after their production. The results are expressed in Colony Forming Units (CFU/g).

Time	Anionic Emulsion	Nonionic Emulsion
T0	10 est	18
T1 environment	< 25	5 est
T1 oven	10 est	500 est
T2 environment	500 est	50 est
T2 oven	1500 est	1000 est

Est: estimated growth.

Thus, only the results of the non-ionic emulsion are not within the established limits, with an average counting of 18.000 CFU/g. The main reason for the high average of microorganisms countings was the absence of a laminar flow and exposure of the sample to atmospheric air in a non-sterile ambient. This is demonstrated by the observation that the results of tests carried out after 30 and 60 days of production, which were conducted in a laminar flow chamber, and presented growth levels within the accepted limits.

The raw materials used in the production of cosmetic formulations are important sources of microbial contamination and therefore must be monitored [25]. Formulations with high water content (like oil-in-water emulsions) and products with higher amounts of water favor microbial growth, it may be presumed that the water used in the formulations and hygiene conditions of the production environment and handler constitute possible sources of contamination [26].

Andrade et al. [27] analyzed 18.241 samples (including raw materials, natural products, water and galenical bases) in relation to microbiological quality. The authors found that 3.5 % of raw materials, 65 % of water samples and 2.8 % of galenical bases used for incorporation of actives) were not in accordance with the specifications of the Brazilian Pharmacopoeia 5th Edition, 2010. Medeiros et al. [28] evaluated nine samples of non-sterile preparations and found that 44.5 % of the samples showed significant values for counting viable microorganisms.

Tonin et al. [29] analyzed the microbiological quality of cosmetic products handled in Planalto Médio-RS. Ten samples

were analyzed, only one exceeded the limits of 1000 CFU/g in the analysis of bacteria. However, six samples exceeded the limits of 1000 CFU/g for fungal growth.

The results denote the need for greater concern and care in implementation of Good Manufacturing Practices, to be implemented at all stages of manufacturing, because the quality of a cosmetic product is incorporated at the same throughout the manufacturing process.

Fungi and yeasts counting

The results of fungi and yeasts count are presented in Table 5. According to the average number of colonies forming units of the samples, it can be seen that the values are within the limits established by RDC No. 481 of September 23, 1999, in established the average recommended for products in topical applications is 1000 CFU/g or mL. It can be observed in anionic emulsion higher growth of microorganisms when stored in the oven (40 ºC), but still, the values lie within the maximum permitted limit.

Table 5. Count of fungi and yeasts in both emulsions after production, 30 days and 60 days after their production. The results are expressed in Colony Forming Units (CFU/g).

Time	Anionic Emulsion	Nonionic Emulsion
T0	< 25	450
T1 ambient	< 25	< 25
T1 oven	500 est	< 25
T2 ambient	100 est	250 est
T2 oven	3050 est	200 est

Est: estimated growth.

Fungi are ubiquitous microorganisms. The high growth of fungi in emulsions for topical use can cause damage, such as superficial infections, when they are applied to the damaged skin and can even cause loss of emulsion stability. The high growth in cosmetics fungi is caused mainly due to storage conditions [29].

Cutaneous mycoses are caused by fungi that infect the superficial keratinized tissue (skin, hair and nails). Among the fungi, the most important are dermatophytes belonging to three genera: *Microsporum*, *Trichophyton* and *Epidermophyton*. Dermatophytoses are among the most prevalent diseases in the world [30].

Conclusion

Emulsions studied in this work were considered acceptable in relation to microbiological parameters, as were within the limits established by RDC No. 481 of September 23, 1999. Thus, it can be stated that the emulsions have acceptable microbiological quality and can be used for topical applications. During the study period, the emulsions were stored in a recipient that is adequate for the storage of these products. It should be noted the importance of conducting other tests such as tests to identify microorganisms pathogenic, tests required to assess the full microbiological quality of the cosmetic product. This work represents a preliminary study on the pharmaceutical development of emulsions for cosmetic use and the data will help to create new perspectives and future studies to obtain cosmetic emulsions, with adequate microbiological quality.

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